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PROLIFERATIVE RESPONSES OF MICE TO A CLONED PLASMODIUM FALCIPARUM SPOROZOITE ANTIGEN

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A peptide fragment of the Plasmodium falciparum circumsporozoite protein (CSP) containing 30 repeats of the immunodominant ASN-ALA-ASN-PRO and two of the VAL ASP variants (R32tet32) is currently being evaluated as a vaccine in man. This R32tet32 peptide, prepared by recombinant DNA technology from a cloned P. falciparum gene fragment, has been examined for its ability to stimulate T-cell proliferation in experimental animals. Groups of mice were injected with either R32tet32 emulsified in Freund's complete adjuvant (CFA), or live, or frozen-thawed P. falciparum sporozoites + CFA. Lymphocytes from such mice were co-cultured with varying doses of R32tet32 or irrelevant antigen. Proliferation was assessed by 3H-thymidine uptake; serum antibody was analyzed by ELISA. A proliferative response was found in mice immunized with R32tet32 + CFA as early as day 7 post-injection, and was persistent through at least day 23. No proliferation in response to R32tet32 was observed in lymphocytes taken from mice injected with live or frozen-thawed sporozoites. All three (continued on reverse)

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19. ABSTRACT (Continued) immunogens induced both IgM and IgG antibody to R32tet32. We conclude that exposure to live or frozen-thawed P. falciparum sporozoites + CFA alone is sufficient to generate T-cell helper activity for subsequent antibody production, but that antigen + CFA was necessary to generate significant T-cell proliferative activity.

Keywords: Malaria · vaccines; immunogens; (KT)

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There has been a worldwide resurgence of malaria, with as many as 300 million cases per year estimated (1). This problem has arisen because mosquitoes have become resistant to insecticides, malaria parasites have become resistant to drugs, and political difficulties have hampered eradication programs. This situation has led to a widespread belief that the main hope for the future is the development of a vaccine (2). Although irradiated sporozoites (spz) of Plasmodium falciparum have been used successfully in human vaccination studies (3,4), conventional mosquito breeding methods would be totally impractical for the large-scale production of sporozoite antigens needed for such a vaccine. Consequently, production of sporozoite antigen using recombinant DNA technology has been proposed and molecular cloning of the gene for the circumsporozoite (CS) protein of P. falciparum has been achieved (5). The amino acid sequence contains a repeated tetrapeptide unit fused with 32 amino acids encoded by a tetracycline-resistance gene read out of frame, and is named R32tet32. Recent studies suggest that the authentic malaria peptide may contain epitopes that are not present in the R32tet32 construct (6). Such information suggests that the R32tet32 peptide may be insufficient to generate an effective T-cell memory response which could be necessary in effective vaccination. Recent results with cholera toxin (7) have demonstrated that certain synthetic peptides are able to prime rabbits to respond to booster inoculation of toxin with a strong neutralizing antibody response, but not to induce neutralizing antibody after primary inoculation alone. These results suggest an additional approach for the testing of cloned peptides as vaccines. The objective of the studies reported in this paper is to investigate the

immunogenic properties of both the R32tet32 peptide and whole P. falciparum spz in mice.

MATERIALS AND METHODS

Mice. BALB/cByJ female mice were obtained from the Jackson Laboratory, Bar Harbor, ME, and housed in laminar flow facilities until used in the experiments presented here.

Antigen. a peptide fragment of the P. falciparum circumsporozoite protein (CSP) containing 30 repeats of the tetrapeptide ASN-ALA-ASN-PRO and 2 of the VAL-ASP variants (R32tet32) was prepared as previously described (5). Preparation and purification of the construct was carried out by A.J. Young and co-workers with Smith Kline French (King of Prussia, PA).

Immunization regimen. Mice were immunized with a single injection in the base of the tail with 20 ug R32tet32 or with the equivalent of 30,000 frozen-thawed P. falciparum spz (strain NF-54) prepared as previously described (8). Both antigens were emulsified at a 1:1 ratio in complete Freund's adjuvant. At times after immunization the mice were anesthetized by inhalation of Halothane, and exsanguinated by retro-orbital sinus puncture. The serum was used for determination of antibody titer as described below. The lymph nodes draining the site of injection (inguinal, popliteal and sciatic) were removed and used in the proliferation experiments described below.

Proliferation assays. Cells from lymph nodes draining the site of antigen injection were prepared by teasing the lymph nodes into Hanks Balanced Salt Solution (HBSS). The cells were adjusted to a concentration of 5×10^6/ml in Eagle's Minimum

Essential Medium supplemented with fetal calf serum, L-glutamine, nonessential amino acids and antibiotics as previously described (9) (CMEM). One hundred ul of the cell suspension was added to each well of a 96-well flat-bottom tissue culture plate (Costar, Cambridge, MA). The wells also received antigen diluted in CMEM to achieve the concentrations noted in the text. At the end of the culture period, the wells were pulsed for 8 hours with 0.4 UCi of ^{3}H-thymidine, specific activity 6.7 Ci/mMole, and the plates harvested on a MASH harvester (Microbiological Associates, Walkersville, MD). The samples were counted in a Beckman liquid scintillation counter by standard methods. Lymph node cells (LNC) to be depleted of T cells were incubated with an optimal dilution of monoclonal anti-Thy 1.2 (ascites fluid from pristane-primed mice injected with the cell line HO 30-12, obtained from the American Type Culture Culture Collection, Rockville, MD). After incubation with antibody on ice for 45 min., the cells were washed twice in HBSS, resuspended in diluted rabbit complement for 30 min. at 37^{o}C, washed, and resuspended in CMEM at the concentration noted above. This regimen was shown to eliminate the ability of LNC to proliferate in response to the T-cell mitogen Concanavalin A (Con A).

Antibody analysis. Enzyme-linked immunosorbent assays (ELISAs) were performed in flat-bottom 96-well Immulon II plates (Dynatech Laboratories, Alexandria, VA). One hundred microliters of R32tet32 (0.1 ugm/ml concentration) in 0.1 M pH 7.5 phosphate buffer was added to each well. The plates were incubated overnight at 37^{o}C and blocked for 1 hour at room temperature with 5 mg/ml bovine serum albumin in borate buffered saline, pH 7.95 (BSA-BBS). The plates were then washed three times with 0.1 mg/ml BSA in borate buffered saline. One hundred microliters of serially

diluted serum was added to the wells and incubated for 2 hours at 37°C. Serum was diluted in 5 mg/ml in BSA-BBS. The plates were then washed as above and 100 ul of horseradish peroxidase (HRP) conjugated goat anti-mouse IgG + IgM (Tago, Inc., Burlingame, CA) diluted in BSA-BBS was added to each well. The plates were incubated for 2 hours at 37°C and washed as above. One hundred microliters of peroxidase substrate (2,2'-azino-di((3-ethyl-benzthiazoline sulfonate) (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) was added to each well and the plates were incubated for 30 min. at room temperature. Color development was measured spectrophotometrically (405 nm) using a Microelisa reader (MR 580, Dynatech Laboratories, Alexandria, VA).

RESULTS

Proliferative responses. In Table I, it can be seen that inguinal and sciatic LNC from mice primed with 20 ug R32tet32 + CFA were able to proliferate in vitro when restimulated with the homologous antigen. LNC taken from mice injected with 30,000 P. falciparum spz + CFA or injected with adjuvant alone (CFA) did not proliferate. These data show that the cloned polypeptide chain is capable of stimulating T-cell immunity when injected into mice. LNC taken from mice injected with 30,000 frozen-thawed P. falciparum spz and restimulated in vitro with R32tet32 were not able to proliferate. These data suggest that, even though the R32 peptide is contained in the P. falciparum sporozoite, it may not be recognized by T cells immune to spz in such a way as to generate a proliferative response to the cloned peptide, or that the dose of 30,000 spz is insufficient.

The data shown in Figure 1 indicate that the proliferative response can be

TABLE I

[2]cpm [3]H thymidine incorporated by LNC taken from mice injected with

[1]Antigen in vitro	ug/well	R32tet32 +CFA (SI)[3]		SPOR + CFA (SI)		CFA (SI)	
R32tet32	5	11,967	(17.0)	872	(1.4)	874	(1.7)
	5×10^{-1}	6,919	(9.8)	997	(1.6)	637	(1.2)
	5×10^{-2}	5,334	(7.6)	561	(0.9)	516	(1.0)
	5×10^{-3}	2,778	(3.9)	748	(1.2)	428	(.08)
Con A	1.25	18,600	(26.3)	15,762	(25.3)	23,269	(45.3)
Media	--	706	--	623	--	514	--

[1]Day 5 in vitro

[2]Plates were pulsed with 0.8 u Ci [3]H thymidine per well 8 hours before harvesting on day 5.

[3]Stimulation index = $\frac{\text{cpm } ^3\text{H thymidine in wells + Ag}}{\text{cpm } ^3\text{H thymidine in wells - Ag}}$

[4]Standard deviation (n=6) <10%

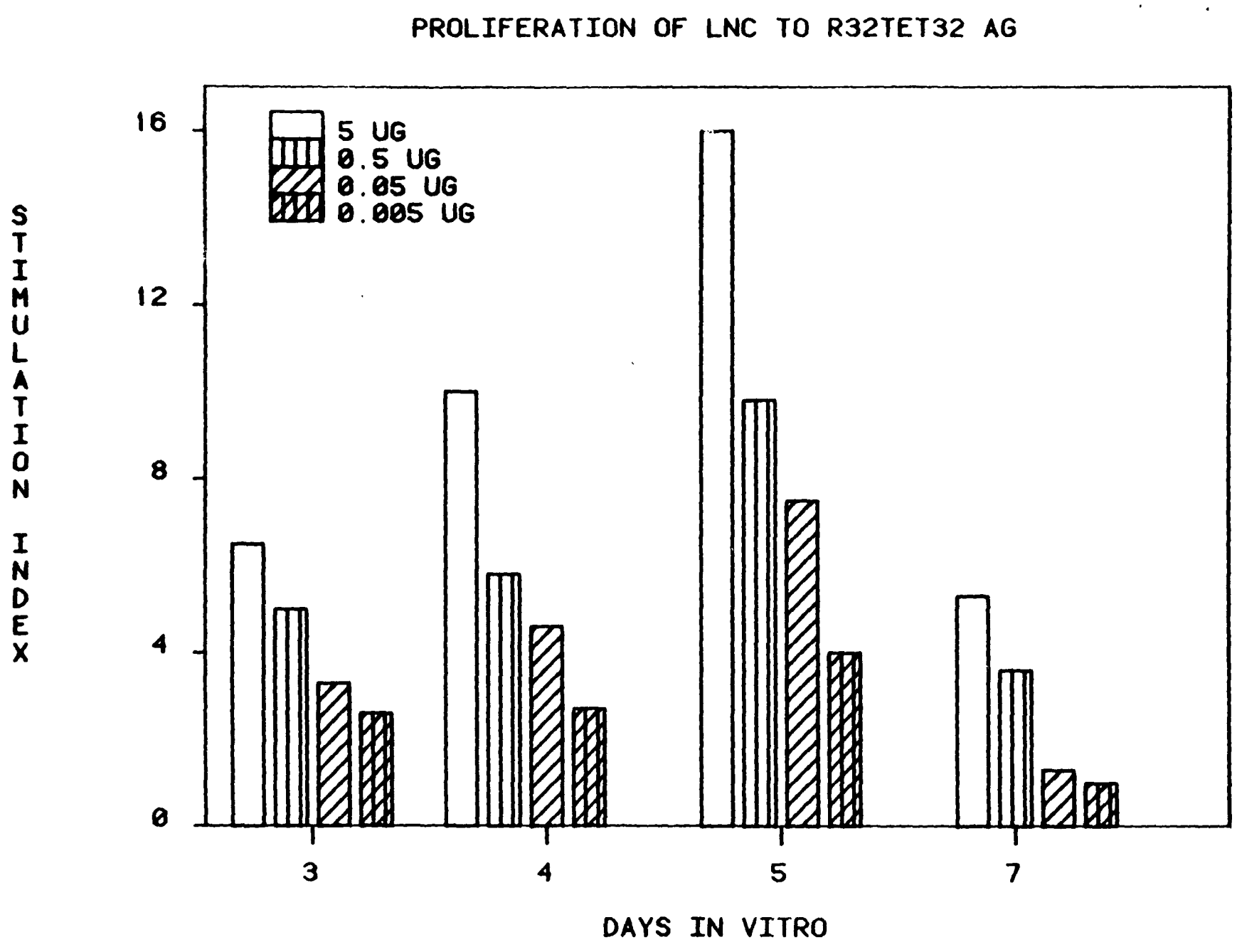
PROLIFERATION OF LNC TO R32TET32 AG
5 UG
0.5 UG
0.05 UG
0.005 UG
16
12
8
4
0
STIMULATION INDEX
3
4
5
7
DAYS IN VITRO

FIGURE 1

demonstrated over a wide range of days in vitro. Significant proliferation can be seen from days 3 through 7, depending on the concentration of antigen in cultures. Additional data (not shown) indicate that in vivo immunization with R32tet32 elicits a long-lasting proliferation response which persists until at least day 23 post-injection.

The data presented in Table II show that proliferation of immune LNC can be reduced by treatment in vitro with monoclonal anti-Thy 1.2 + C. That the control proliferation in response to Con A is reduced but not eliminated suggests that not all T cells were eliminated with one cycle of antibody and C treatment. Again, LNC taken from mice injected with adjuvant alone did not proliferate in response to the R32tet32 peptide.

Antibody responses. The data in Figure 2 show that mice injected with either the cloned R32tet32 + CFA peptide or P. falciparum spz + CFA developed antibody to the R32tet32 peptide. This is in contrast to the data presented above, in which mice injected with spz did not develop a proliferative response to R32tet32. The data presented in Figure 2 were developed using a secondary reagent which recognizes IgM and IgG; similar results were obtained when using secondary reagents directed to either mouse IgM or mouse IgG (data not shown). These results suggest that the dose used in the CSP antigen present on the spz is capable of stimulating an antibody response to R32tet32, but cannot stimulate a proliferative response to the peptide produced by recombinant means.

DISCUSSION

The role of T cells in resistance to malaria in both humans and experimental animals has been the subject of intense study. Current theories support a helper role

TABLE II

[2]cpm [3]H thymidine incorporated by LNC taken from mice injected with

[1]Antigen in vitro	ug/well	Treatment of LNC[2]	R32tet32 + CFA	CFA
R32tet32	5	Anti-Thy 1.2 + C'	710	2,314
	5×10^{-1}		346	748
	5×10^{-2}		384	875
Con A	1.25		4,259	684
Media	--		208	100
R32tet32	5	C' Alone	l2,732	2,850
	5×10^{-1}		4,141	814
	5×10^{-2}		1,545	852
Con A	1.25		64,040	56,269
Media	--		933	788

[1]Conditions as described in Table I.

[2]Cells were treated with monoclonal anti-Thy 1.2 + C' by standard methods. Cell numbers were adjusted to the original concentration and plated as described in Materials and Methods. Proliferative responses to Con A were reduced by 93% after this treatment.

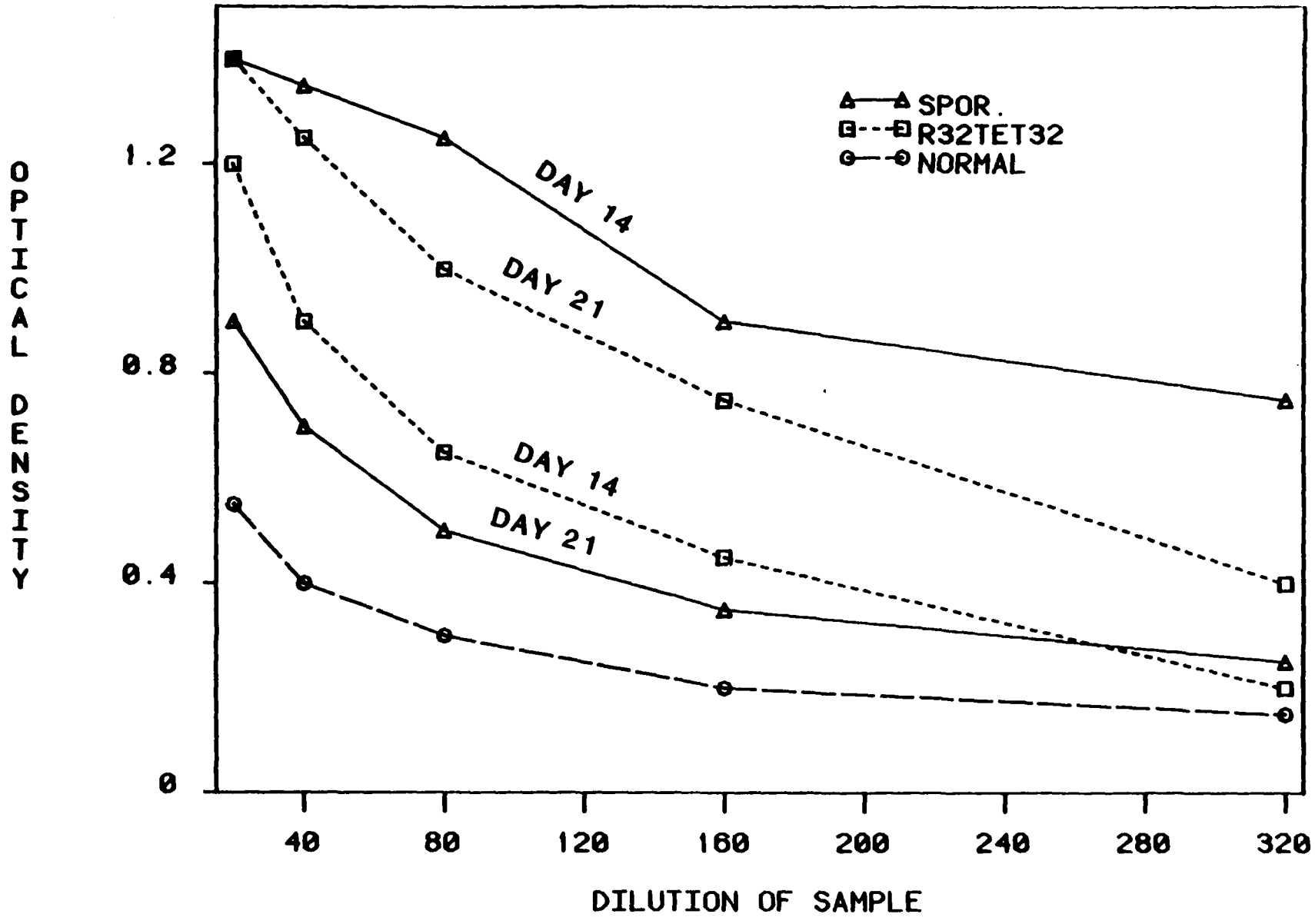
SPOR.
R32TET32
NORMAL
DAY 14
DAY 21
DAY 14
DAY 21
OPTICAL DENSITY
1.2
0.8
0.4
0
40
80
120
160
200
240
280
320
DILUTION OF SAMPLE

FIGURE 2

for T cells in the clearance of rodent malarias, since both T cells and B cells are necessary to confer immunity to malaria in mice (10). In recent experiments, antigen-specific T cells were examined for their ability to reconstitute T-cell-deprived mice for resistance to infection with murine malaria species. It was shown that antigen-specific T-cell clones or bulk cultures were able to restore anti-malaria capacity in such mice. These T cells apparently acted indirectly as a helper or amplifier cell to confer resistance (11).

It has not been possible to show a protective role for antigen-specific T cells in our model system, since P. falciparum is not infectious in mice. Our results would suggest, however, that T cells recognize the peptide antigen R32tet32 both in the efferent and afferent arms of the immune response. These stimulated T cells were able to confer help, as demonstrated by the anti-R32tet32 IgG response generated (not shown).

The fact that P. falciparum spz, whether administered live by the intravenous route or emulsified in CFA and administered subcutaneously, stimulated an antibody response but not a T-cell proliferative response is a different finding to analyze. This may reflect differences in immunization requirements in the mice or a difference in the relative densities of relevant epitopes in spz vs peptide antigens. While it is well known that the immunization requirements for activation by antigen differ between T cells and B cells (12-14), the fact that an antibody response could be demonstrated in sporozoite-injected mice in the absence of a proliferative response suggests that other mechanisms of immunity might be involved. Since malaria infection has been shown to suppress immune responses in a variety of experimental systems (15), the possibility that spz are inducing a suppressor effect cannot be ruled out by the findings

presented here. This subject is currently under study in our laboratory. A simple, though unattractive, explanation may be that the LNC from mice injected with R32tet32 are proliferating in response to the 'nonsense' peptide generated by out-of-frame reading of the tet32 portion of the R32tet32 peptide. Alternatively, antibody cross-reactivity between antigens contained in spz and those expressed by the R32tet32 peptide may explain these differences; the R32tet32 may contain an epitope(s) which is recognized by antibodies elicited by sporozoite antigens, but which is not contained in the T-cell stimulating fragment. T cells have been shown to be exquisitely sensitive to single amino acid substitutions, thereby allowing analysis of the fine specificity of restimulation to peptide antigens. Further, in the sperm whale myoglobin system, antigenic sites which bind antibody are sterically separated from those which stimulate T-cell proliferation (13). Since T cells are sensitive to small changes in amino acid sequence, an immunization protocol involving spz may not sensitize T cells to R32tet32, while stimulating an antibody which reacts with R32tet32. We are currently generating T-cell lines which may distinguish among these possibilities.

FIGURE LEGENDS

FIGURE 1.

1. LNC from mice injected with R32tet32 were incubated with antigen or with medium alone for the number of days indicated in the Figure. Four identical plates were set and harvested at 24-hour intervals.

2. Data are expressed as stimulation index, where

$$S.I. = \frac{\text{cpm LNC + Ag}}{\text{com LNC - Ag}}$$

FIGURE 2.

1. ELISA assay performed on pooled serum samples taken from mice injected with either spz + CFA (spor), R32tet32 + CFA (R32tet32), or with CFA alone (normal). Mice were bled on the days following injection as indicated.

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